Non-catalytic *in-situ* (trans) esterification of lipids in wet microalgae *Chlorella vulgaris* under subcritical conditions for the synthesis of fatty acid methyl esters

Charles Felix[a,i], Aristotle Ubando[a,i], Cynthia Madrazo[b,i], Ivan Henderson Gue[a,c], Sylviana Sutanto[d], Phuong Lan Tran-Nguyen[e], Alchris Woo Go[f], Yi-Hsu Ju[d], Alvin Culaba[a,i,*], Jo-Shu Chang[g,h,j,k], Wei-Hsin Chen[l]

[a] Mechanical Engineering Department, De La Salle University, 2401 Taft Ave., Manila 0922, Philippines
[b] Chemical Engineering Department, De La Salle University, 2401 Taft Ave., Manila 0922, Philippines
[c] Mechanical Engineering Department, Far Eastern University Institute of Technology, P. Paredes St., Sampaloc, 1015 Manila, Philippines
[d] Department of Chemical Engineering, National Taiwan University of Science and Technology, 43 Keelung Rd. Sec. 4, Taipei 106-07, Taiwan
[e] Department of Mechanical Engineering, Can Tho University, 3-2 Street, Can Tho City, Viet Nam
[f] Department of Chemical Engineering, University of San Carlos, Talamban Campus, Gov. M. Cuenco Ave, Nasipit, Talamban, Cebu City 6000, Philippines
[g] Department of Chemical Engineering, National Cheng Kung University, 1 University Rd, East District, Tainan 70101, Taiwan
[h] College of Engineering, Tunghai University, Taichung 407, Taiwan
[i] Center for Engineering and Sustainable Development Research, De La Salle University, 2401 Taft Ave., Manila 0922, Philippines
[j] Research Center for Energy Technology and Strategy, National Cheng Kung University, Tainan 701, Taiwan
[k] Research Center for Circular Economy, National Cheng Kung University, Tainan 70101, Taiwan
[l] Department of Aeronautics and Astronautics, National Cheng Kung University, Tainan 701, Taiwan

HIGHLIGHTS

- Biodiesel was produced directly from microalgal biomass with 80% moisture content.
- Design of experiment using definitive screening that has relatively fewer runs.
- Optimization of parameters using the biodiesel yield and process power consumption.
- Biodiesel yield reached as high as 74.6% with respect to the maximum amount of oil.
- Compared the process parameters to its conventional transesterification counterpart.

ARTICLE INFO

ABSTRACT

Microalgae offer promising and multifaceted solutions to the ongoing issues regarding energy security and climate change. One of the major bottlenecks in utilizing algal biomass is the excessive amount of moisture to be managed after harvest, which translates to costs in the dewatering step. Newer strategies have been developed to be able to convert algal biomass feedstock to biodiesel without the need for extraction and drying, such as in-situ transesterification. This process can be improved by concurrently subjecting the system under subcritical conditions, which could also potentially remove the use of catalysts as well as offer tolerance to free fatty acid content of the feedstock. A definitive screening design of experiment was utilized to provide an acceptable prediction on the effects of key process parameters – temperature, reaction time, and solvent-to-solid ratio to the obtainable fatty acid methyl ester (FAME) yield and process power consumption. The optimum operating condition, which combines the benefits of maximizing the FAME yield and minimizing the process power consumption was found to be at 220 °C, 2 h, and 8 ml methanol per gram of biomass (80 wt% moisture). This produces a FAME yield of 74.6% with respect to the maximum obtainable FAME. Sensitivity analysis discussed the implications regarding the weight of importance between the two responses of interest. The benefits of the proposed process can be observed when compared to its conventional transesterification counterpart in terms of energy savings and reduced environmental impact. Hence, this process offers a feasible alternative to produce biodiesel from microalgae.

* Corresponding author at: Mechanical Engineering Department, De La Salle University, 2401 Taft Ave., Manila 0922, Philippines.
E-mail address: alvin.culaba@dlsu.edu.ph (A. Culaba).

1. Introduction

It is generally accepted that renewable energy has an indispensable role in terms of addressing the ongoing issues of energy security and climate change, whether at a domestic or global scale. Numerous countries have already taken initiatives in slowing down fossil-based energy usage and its accompanying environmental impacts by accelerating investments in alternative energy sources. While there is a steady rise of renewable energy technologies being investigated, reliance on fossil-based energy is still paramount [1]. In the transport sector, years of technological improvements still keep the overall oil demand on a rising trend. To promote the use of biofuels, the choice of feedstock should steer clear from conventional food-based sources such as palm and soybean, as well as from biological wastes and residues [2]. Another important factor to consider is the overall cost of production which is often the selling point of renewables to stakeholders.

Research efforts have been devoted to the development of processes to utilize versatile biomass feedstock like those of microalgae [3]. Microalgae are unicellular photosynthetic microorganisms typically found in freshwater and marine systems and are capable of producing multiple and high-value chemical compounds [4]. In producing biomass from microalgae, the upstream processing also presents numerous promising benefits including carbon sequestration [5], wastewater treatment potential [6,7], minimum and non-arable land requirement, fast growth rates [8] and very high oil yields [9]. Depending on the conditions during its cultivation stage, there is an array of options in converting biomass into useful bioenergy [10,11]. A great deal of investigations on microalgal biomass applications are relatively more complex and economically expensive in relation to the status quo of fossil-based fuels [10]. However, many have argued that there are still grounds for optimism to develop better and more sustainable processes that take advantage of this prospective biomass [8,12]. The aspect of utilizing the oil fraction of microalgal feedstock has been well-established [13]. However, due to the cultivation requirements in the biomass production, the moisture content after harvest can be very difficult to manage. The moisture removal through conventional drying process is considered as a bottleneck due to the significant amount of energy consumed [8]. Apart from the energetic requirements of the aforementioned process steps, free fatty acids (FFA) present in the biomass are fairly high and are affected by the nature of the feedstock and storage conditions [14].

Research efforts are now thrusted towards exploring relatively novel approaches and technologies which are directed to reduce the cost and energy consumption of biodiesel production from microalgae [13]. A direct or in-situ transesterification approach is one of the potentially viable biodiesel production process, which involves both extraction and transesterification reaction to commence in a combined reactive-extraction environment [15]. One considerable advantage of the in-situ approach is that all of the lipids present in the biomass can be made into contact with the solvent, thereby eliminating a discrete extraction step which incurs oil losses depending on the solvent/s and/or co-solvents used [16]. In addition, the oil recovery and refinement stages prior to the actual biodiesel conversion step can be excluded [15]. The conversion aspect also includes the esterification of free fatty acids apart from transesterification of triglycerides, which presents an opportunity to improve the overall fatty acid methyl ester (FAME) yield from the chosen biomass [17].

Even though in-situ transesterification renewed the interest of many in the pursuit of a robust and sustainable biodiesel source, there are still some setbacks which have yet to be addressed in order to reap its benefits. In relation to the conventional transesterification approach, more resource inputs in the form of solvents and catalysts are needed

for the reaction to occur especially if samples with relatively higher moisture contents are utilized [18]. Many studies adapting the in-situ production route investigated various types of catalysts [19,20], co-solvents [21] and observed their corresponding effects to the obtainable FAME yields. Various technological approaches were also integrated such as ultrasound and microwaves [22,23]. Nonetheless, these processes have failed to anticipate the possibility of scaling up. For instance, the use of solvents and catalysts necessitate for possible purification step/s which add to the operating costs. Reactions involving the use of microwaves may be hurdled by the readiness of construction materials and possible safety reasons [15]. Hence, it is highly favorable to use approaches that can handle the water present in the harvested microalgal biomass with minimum resource inputs and simplified production processes and equipment requirements.

While the presence of moisture imposes some issues in itself during the reaction as an inhibitor or diluting agent between the interaction of the solvent in the solid matrix [16], some studies have actually rendered moisture to be rather tolerable or even useful [24,25]. These processes subject their systems at a relatively high temperature (above 120 °C) and pressures (up to 20 MPa), as in sub- and supercritical conditions. In either approach, the critical point of the mixture between the oil in the biomass, solvent, and moisture is estimated to be near the critical point of the solvent since it is the predominant reactant in the system [15]. Operating at supercritical conditions greatly improves the solubility of oil in the solvent as they became a single phase, resulting in faster completion of the reaction and high tolerance to not only moisture but also free fatty acid content of the feedstock [26,27]. However, the severity of the process parameters will have drawbacks regarding energy consumption, methanol loading, thermal degradation of biodiesel products and the cost of the apparatus itself [28,29]. Pressurized water at subcritical state having a temperature between its boiling and critical points also offers benefits in the outcome of direct transesterification. Under subcritical conditions, water can act as a non-polar solvent and while having increased ionization, allows it to aid in the extraction process and serve as a pseudo-catalyst, respectively [30,31]. Further, the presence of water under subcritical conditions also facilitates the hydrolysis of the cellular matrix and available lipids, both storage and structural, whereby permitting the stored polar organic compounds such as phospholipids is made possible under such conditions [24].

Numerous studies have tackled the application of direct transesterification under either sub- or supercritical conditions making use of a wide variety of feedstocks, including less desirable ones such as microalgae [24,32] and activated sludge [31]. Many of these studies were rather exploratory, which are often executed using one-factor-at-a-time experiments to obtain a suitable operating condition. A major disadvantage of using such approach is the lack of capability to establish relationships among numerous variables by means of models. The simultaneous effects of the considered parameters were also not possible to be observed. Moreover, only the product yields of these processes were monitored accordingly by these studies, and as such maximizing the biodiesel yield has only been the sole parameter in determining the best operating conditions. The performance of these processes can be further optimized by not only maximizing the desired factors such as the FAME yield, but also by minimizing a few undesired factors as well such as the energy requirement, which is a major consideration to the process. The tradeoff between these two process metrics have not been evaluated by related works.

This work aims to provide an improved approach in determining the optimal operating conditions of the subcritical in-situ transesterification environment using naturally wet biomass feedstock such as microalgae. Using a relatively new design of experiment platform such as

definitive screening design, the development of mathematical models regarding the effects of factors can be estimated and possibly optimized with significantly fewer trials [33]. For this work, an additional response of interest was also introduced and integrated in the model synthesis, which is the total power consumption of the process, a metric which should also be given equal importance with the biodiesel yield. This work is, by far, the first to examine the implications of the power consumption on the selection of the optimal operating conditions of such processes.

The apparent benefits offered by the in-situ biodiesel production route over the conventional pathway have not been evaluated extensively as well. For instance, the reduction in process steps offered by the in-situ route has not been translated effectively into savings, especially in terms of energy. This bears importance especially in evaluating the sustainability aspect of the proposed process of bioenergy production. As stated earlier, the amount of solvent loading (mostly methanol) when performing in-situ transesterification is significantly more than what is generally required with the conventional approach. The operating temperatures and pressures will also become considerably higher when subjecting the system under either sub- or supercritical conditions. For it to become a sustainable net producer of energy, these setbacks should be dominated heavily by the supposed benefits. In this regard, this work also aims to shed light on the matter on a lab-scale basis, by comparing the environmental footprint and energy demand of the two aforementioned process pathways.

The focus of this study is the direct transesterification utilizing *Chlorella vulgaris* biomass with high moisture content (80 wt%) under subcritical conditions. Experiments were conducted following a definitive screening design platform to investigate the effects of reaction temperature (200–250 °C), reaction time (1–3 h), and solvent-to-solid ratio (2–8 ml methanol per gram of wet biomass weight). The yield was quantified by gas chromatography, and the reaction was monitored for its process power consumption to obtain the actual energy input required by the process.

2. Materials and methods

2.1. Materials

Freeze dried *Chlorella vulgaris* were obtained from National Cheng Kung University, Taiwan. Prior to being used, the biomass was transformed into fine powder by grinding and were stored at −20 °C freezer. The methanol used for the transesterification process were of HPLC grade. Other reagents such as n-Hexane were of industrial grade. For the profiling of fatty acids of biodiesel product, a 37 FAME component mix, and oleic acid (C18:1), both obtained from Supelco (Bellefonte, PA), were used as standards for gas chromatography analysis. For monitoring of the power consumption for each trial, a power cost monitor (PowerPro 2022) from Prodigit Electronics Co. Ltd. was used to give the actual kWh readings.

2.2. Biomass characterization

The samples were analyzed according to the quantity (crude lipid content) and quality (wax & gum, saponifiable matter, and free fatty acid) of its lipid content.

2.2.1. Moisture

To check the initial moisture content of the samples received, the water content present was calculated by simple weight difference before and after freeze drying. The final moisture content was obtained after constant weight after three trial periods.

2.2.2. Crude lipids

A simple solvent extraction employing an equi-volume amount of methanol and hexane was adapted based on the results of [34]. Two grams of dried *Chlorella vulgaris* and 150 ml of solvents were mixed in a tightly capped glass bottle and was subjected to a water bath at 60 °C and with 350 rpm magnetic stirring. After 4 h extraction period, vacuum filtration separated the solid particles. To promote phase separation, the filtrate was transferred to a separatory funnel where 20 ml salt solution with concentration of NaCl of 5 wt% was supplied. The upper layer which contained the crude lipids were manually obtained. Hexane was vacuum evaporated and the lipids were purged under nitrogen until constant weight.

2.2.3. Wax, gum, and free fatty acid (FFA)

An estimated 0.5 g of extracted oil and 5 ml of acetone was stirred in a water bath (60 °C) for 1 h. The mixture was cooled to room temperature and then refrigerated (4 °C) for 3 h similar to the procedure
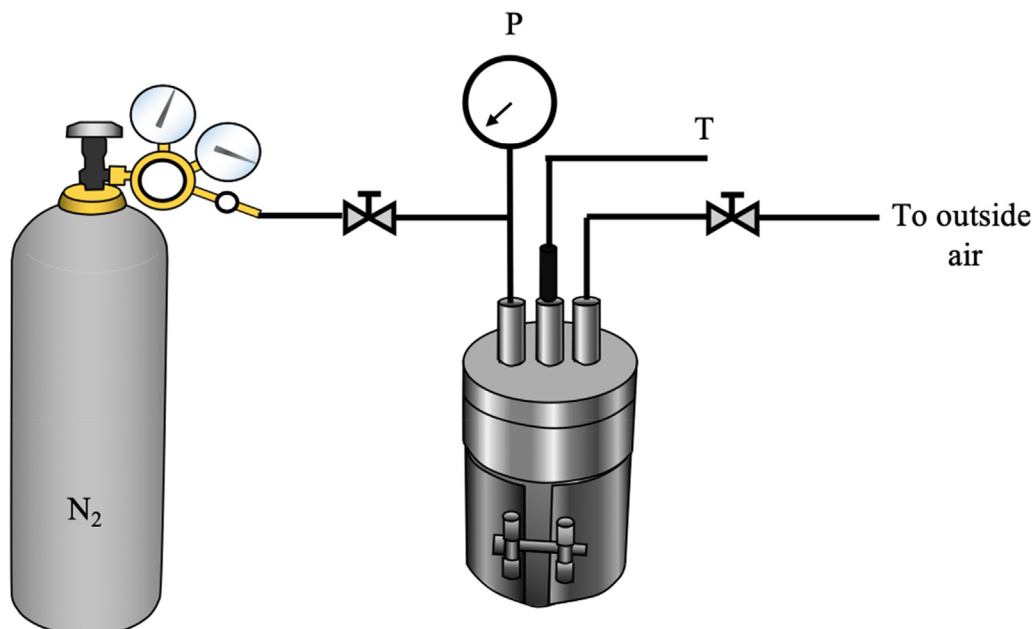

Fig. 1. Basic schematic of the reactor setup.

done by [14]. The cold mixture was passed through a membrane filter (0.22 um pore size). This procedure was repeated twice to ensure complete removal of wax and gum. The dewaxed and degummed oil was injected to gas chromatography to determine the specific amounts of FFA, mono-, di-, and triglycerides.

2.2.4. Saponifiable and unsaponifiable matter

In a screw-capped bottle, 5 ml of ethanol were mixed with 0.5 g of extracted crude oil. Based from AOCS official methods (Method 6b-53), Around 0.3 ml of 50% KOH was added before placing the system on a water bath at 80 °C for 3 h. The mixture was then transferred to a separatory funnel where 10 ml deionized water and 10 ml hexane was added to promote phase separation. The lower saponifiable layer was released and the upper unsaponifiable layer was rinsed with 0.05 N KOH for three washings. Afterwards, the upper layer was collected, and hexane was evaporated until constant weight to obtain the amount of unsaponifiable matter.

2.2.5. Fatty acid profiling

The lower layer from the previous sub-section was hydrolyzed into free fatty acids by addition of concentrated H_2SO_4 until the ph becomes less than 2, similar to the work of [14]. The mixture was stirred for 3 h before transferring it into a separatory funnel. Adequate amounts of hexane and 5 wt% NaCl were added to separate and collect the free fatty acids. Hexane was evaporated to obtain pure FFAs, which were then reacted with boron trifluoride-methanol solution under 60 °C water bath and adapting the aforementioned filtration procedures. The organic product was subjected to gas chromatography to generate a profile of various fatty acids present. This was used to compare the fatty acid profile before and after the subcritical transesterification reaction.

2.2.6. Subcritical water pretreatment

Since chloroform was not used due to its hazardous nature, some of the samples underwent cell disruption by subcritical water (SCW). Dried biomass was added with water to obtain 90 wt% water content and was loaded in the reactor, illustrated in Fig. 1. The samples were subjected under 175 °C with corresponding pressure of around 500 psi for 10 min [35]. After pretreatment, the products were aggregated when the reactor reached room temperature. The products were freeze-dried and the same characterization methods, as discussed previously, were applied to the pretreated samples as well.

2.3. Design of experiment (DoE)

A lot of determinants affect the outcome of in-situ transesterification of feedstocks. These include the reaction temperature, time, pressure, agitation, as well as the type and amount of reactants used [15,36]. Three important factors were selected as variables for this study – reaction temperature, reaction time, and solvent-to-solid ratio, as they are easily controllable and continuous. Under subcritical conditions, this work is the first to examine this combination of factors and in a design of experiment approach. Definitive screening design (DSD) was chosen as the platform to carry out the experiments as this enables the evaluation of the aforementioned factors at three levels (low, medium, high) and provide an estimate of optimal parametric setting at a significantly reduced number of experimental treatments. Definitive Screening Design can also approximate the effects of the main and quadratic terms for each factor without confounding [37].

The actual values for the three factors were reaction temperature – 200 °C, 225 °C, and 250 °C, reaction time – 1 h, 2 h and 3 h, and solvent-to-solid ratio of 2, 5, and 8 ml methanol per gram of wet biomass. Two responses were identified as the FAME yield (%) and the process power consumption, as explained in the previous section. The former was expressed as the weight of FAME obtained per unit weight of dry biomass used, while the power consumption of the reactor was the obtained kWh reading for the entirety of the reactive extraction process.

Previously reported studies related to this work that have performed DoE have only focused on the former response alone [30,38]. Power consumption was considered as an equally important response with the yield since the process parameters under this condition are significantly higher relative to its conventional counterpart.

JMP Pro 11 was the software utilized to generate a randomized DSD runs table and implement the statistical analysis. To ensure reproducibility of the results, each trial was performed twice and the representative value for the responses were taken as the mean of the two data points. The results of the DoE were tested for regression using a response surface model consisting of the main, quadratic, and interaction terms for each factor as also done in the work of [39]. The significance of each term was tested using derived *p*-values which implies the term was significant if the respective *p*-value fall below 0.05.

2.4. In-situ transesterification under subcritical conditions

Wet feedstock (80 wt% moisture) was generated by combining 1 g of the freeze-dried biomass and 4 g of deionized water in a glass chamber. After adding a predetermined amount of methanol, the system was loaded inside the same reactor (Fig. 1) having a working volume of 175 ml [32]. After sealing and insulating, the reactor was placed on top of a magnetic stirring plate and air was removed inside by purging nitrogen gas for a few minutes. Electric heating was supplied at a rate of 5–6 °C which translates to approximately 40–45 min heating time as also observed in related works [14]. A thermocouple integrated with the reactor was used to keep the system temperature within ± 2 °C range for the entirety of the reaction period. After a predetermined time, the reactor was cooled until below the normal boiling point of methanol. Nitrogen gas was released by opening the pressure valve and the products of the reaction were collected accordingly.

Crude biodiesel product was separated from its solid components using a Buschner funnel setup making use of an 8 μm pore size filter paper (Advantec No. 2). The unreacted methanol was evaporated from the filtrate and the products were transferred to a separatory funnel, where the crude biodiesel was collected by adding 5 wt% NaCl solution and extracting the organic phase with hexane. The final weight of the product was acquired by collecting the upper layer and evaporating hexane until constant weight. The collected biodiesel product was checked for its purity via gas chromatography (GC).

The samples for gas chromatography were prepared by vortex-mixing of ethyl acetate and crude biodiesel with approximate amounts of 1 ml and 18 mg, respectively. Anhydrous magnesium sulfate was used in a hydrophilic PVDF membrane (0.2 μm pore size; ChromTech) to filter the solution. An aliquot of 1.5 μm was injected into the GC-FID (Shimadzu GC 2010, Kyoto, Japan) for FAME qualitative and quantitative analysis. Among other evaluation includes the quantification and of other unreacted oils such as mono-, di-, and triglycerides, and free fatty acids. The separation column was a nonpolar ZB-5HT (5% phenyl) – methylpolysiloxane column (15 m × 0.32 mm ID, 0.1 μm film thickness) sourced from Phenomenex, USA. Key GC working parameters were set using Shimadzu GC Solution v 2.3 Software. Injector and detector temperatures were at 370 °C, the column temperature started from 80 °C and was increased at a rate of 15 °C/min to 365 °C and held for this temperature for 10 min. This translates to a total run time of 29 min. Flow rates of gases were set as: hydrogen − 50 ml/min, air − 500 ml/min, and makeup flow − 30 ml/min. Nitrogen, which was the carrier gas, was at 30 cm/s at 80 °C.

2.5. Comparative impact assessment

The aim of this assessment is to compare the impacts of the proposed in-situ transesterification process under subcritical conditions to the conventional biodiesel production process which had discrete stages for drying, oil extraction, and oil conversion. A life cycle assessment (LCA) framework was conducted for quantifying the global warming

potential (GWP), and the cumulative energy demand (CED) for both process pathways. One gram of FAME was the chosen functional unit. The in-situ pathway considered the actual reaction under subcritical conditions which employed data obtained from the optimized experimental trial in this study. Under the conventional route, separate lab-scale experiments were performed for the freeze drying, and oil extraction steps using the same material, and a base-catalyzed transesterification which had 94 percent conversion efficiency was adapted based from the results of [40].

The system boundaries for the conventional and in-situ transesterification pathways are illustrated, respectively, in Figs. 2 and 3. For the quantification of the impacts, both pathways were evaluated focusing on the downstream processing, which was the conversion of wet harvested biomass into FAMEs. The common stages of cultivation and harvesting, as well as the end-use of the biodiesel product were assumed to be similar for the compared pathways and are thus intentionally excluded in the scope of the assessment to further highlight the benefits offered by the in-situ route. In the work of Chaudry et al. [41] less important stages of the well-to-wheel life cycle were also excluded in the system boundary to highlight the benefits of one method and compare it with its conventional counterpart.

External material streams of the system boundary consist of hexane, methanol, and methoxide while the external energy stream consists of the electricity consumption throughout the process. Data regarding the emissions and material consumption of the external streams were obtained from the inventory database EcoInvent. The characterization of the GWP from emissions and material consumption was quantified according to the impact assessment EDIP 2003, while the energy demand of the external streams was calculated via the Cumulative Energy Demand (CED) impact assessment. SimaPro 8.5.2.0 was the software used for this part of study.

3. Results and discussion

3.1. Biomass characterization

Key lipid characteristics of *Chlorella vulgaris* biomass with and without the addition of SCW pretreatment are enumerated in Table 1. The initial moisture content of the sample was around $1.65 \pm 0.8\%$. The total amount of crude lipids that can be extracted was determined by organic solvent extractions. For this purpose, numerous studies have utilized chloroform-methanol mixtures. However, as the former solvent is known to be very toxic, an equi-volume amount of hexane and methanol was utilized instead as an alternative [34]. In addition, water at subcritical state has also been used recently as a pretreatment step which is helpful in increasing the obtainable lipids [35]. These approaches were applied to better approximate the maximum theoretical crude lipids present from the biomass samples without using chloroform.

The use of subcritical water as a pretreatment step substantially increased the maximum extractable amount of lipids by as much as 35%. The significant increase suggests that the pretreatment method was able to disrupt very well the rigid cell walls of the algal cells which contained most lipids. Furthermore, this type of pretreatment greatly reduced the composition of unwanted wax and gum which could signify that they were broken down to release its constituent fatty acids which will contribute to higher FAME yields [31]. In the conventional route, these components (wax esters and phospholipids) were often not included in the conversion process and thus, the available fatty acids that could be used in the synthesis of FAMEs were not maximized.

The fatty acid profile of the *Chlorella vulgaris* feedstock is listed in Table 2 with the corresponding chromatogram shown in Fig. 4. The predominant fatty acids were composed of Oleic (C18:1), Palmitic (C16:0), Palmitoleic (C16:1), and Stearic Acid (C18:0). From Table 2, it is apparent that most of the FAMEs synthesized were unsaturated. The composition of fatty acids behaved similarly to the obtainable crude

biodiesel in all experiments.

3.2. Effects of factors

The resulting FAME yields and the respective power consumption of the reactor derived from varied treatments of reaction temperature, time and the solvent-to-solid ratio (SSR) are listed in Table 3. The recorded FAME yields ranged from around 5–14% proportionate to the amount of dried biomass. If the yield is expressed with respect to the maximum theoretical FAME attainable (obtained by subtracting the amount of unsaponifiable matter to the total crude lipids), the yield translates to about 28–74%. Meanwhile, the power consumption ratings recorded ranged from 0.33 to 0.74 kWh.

The Prediction Profiler for the three factors, showing the results of the central treatment values of the three factors to the two responses is presented in Fig. 4.

3.2.1. Reaction temperature
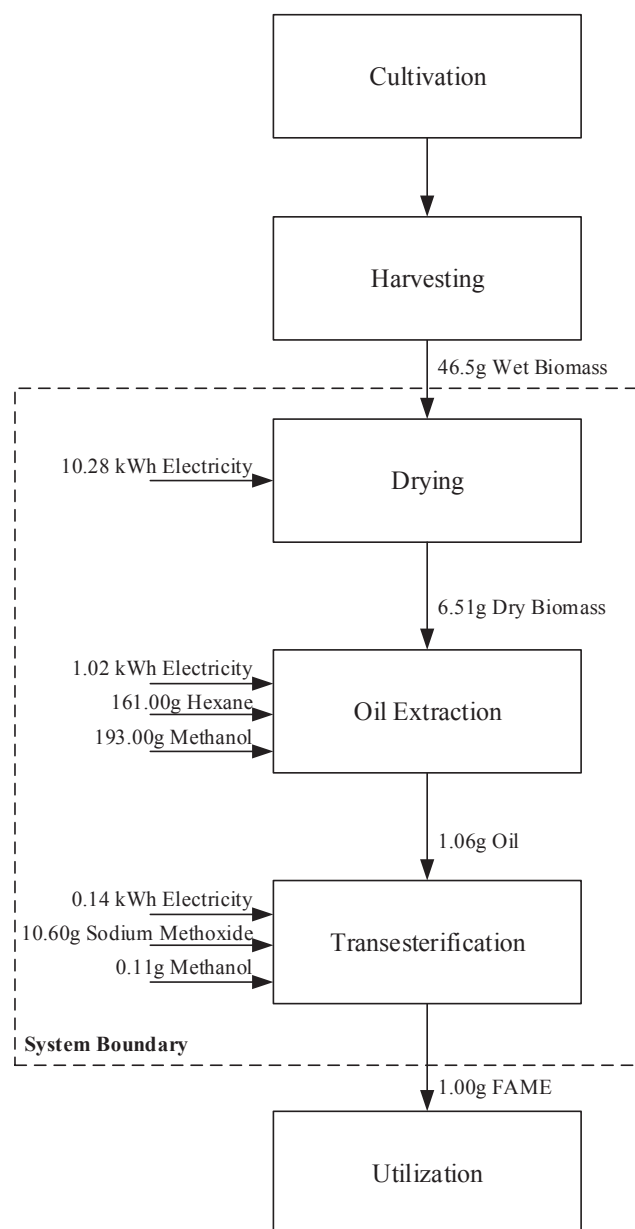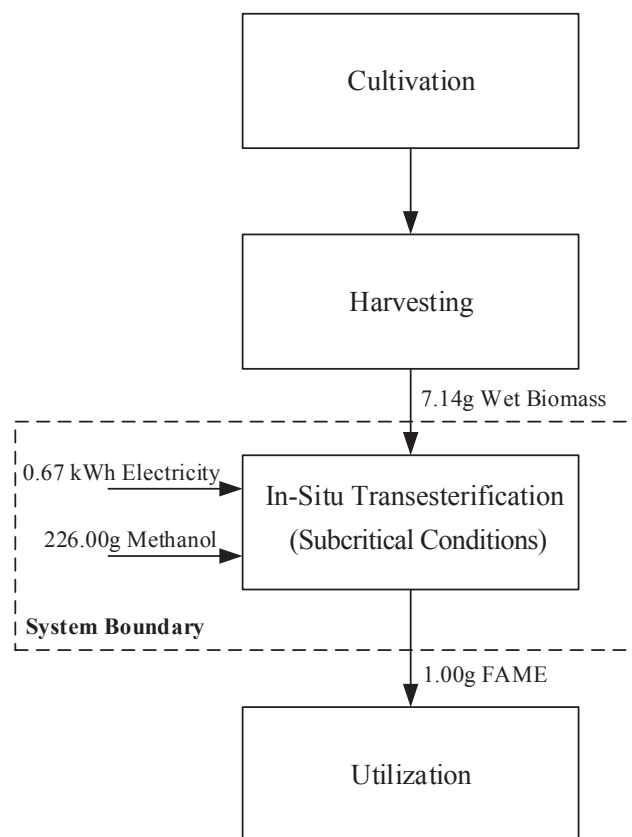
From Fig. 5, A positive correlation between the reaction

Fig. 2. System boundary for conventional transesterification pathway.

Fig. 3. System boundary of in-situ transesterification (under subcritical conditions) pathway.

Table 1
Characteristics of dry *Chlorella vulgaris* samples before and after subcritical water pretreatment.

Component (%)	Without pretreatment	With pretreatment[*]
Crude lipids[b]	16.33 ± 0.24	22.08 ± 0.53
Wax and gum	12.16 ± 0.46	2.07 ± 0.46
Unsaponifiable	0.835 ± 0.12	3.23 ± 0.86
Saponifiable	89.82 ± 1.01	96.37 ± 0.79
Free fatty acids	8.52 ± 0.79	> 30

* 175 °C, 10 min [35].

Table 2
Fatty acid profile of *Chlorella vulgaris* biomass (as % of methyl esters) under non-catalytic subcritical in-situ transesterification.

Fatty acid	Name	% in sample
C14:0	Myristic acid	0.18 ± 0.04
C16:1	Palmitoleic acid	15.90 ± 0.28
C16:0	Palmitic acid	22.38 ± 0.65
C18:3	Linolenic acid	0.51 ± 0.04
C18:2	Linoleic acid	4.39 ± 0.42
C18:1	Oleic acid	48.05 ± 0.1
C18:0	Stearic acid	8.59 ± 0.49
	Saturated fatty acids	31.15 ± 1.13
	Unsaturated fatty acids	68.85 ± 0.76

temperature was observed such that an increase in operating temperature favors the formation of FAMEs, but only up to certain levels of operation. This observation is in agreement with other published works which utilized other feedstocks aside from microalgae [24,30,31]. Higher temperatures permit better solubility of the reacting oil with the solvent with water at subcritical state assisting in the breakdown of

lipid components before reacting with methanol. The improved solubility of the oil content of the feedstock with water is often associated with decreasing dielectric constant of the solvent which was found to reach the dielectric constant of acetone [15]. The amount of moisture present also aided in the reaction as a hydrolysis agent [42,43]. For this study, the reaction temperature started to produce the same amount of yield starting at around 220 °C.

An increase in the working temperature also effected a steady rise in the power consumption of the process as the higher operating temperature would require more heat, and consequently more power, in order to attain it. The power consumption can thus be appropriated for the heating period of the reactor up to the desired working temperature.

3.2.2. Reaction time

Similar to the reaction temperature, a longer reaction period (including the ramp-up time to the working temperature) also resulted in a positive nonlinear increase in the biodiesel yield. This holds true up to a certain level where the system reaches equilibrium as well. Based from Fig. 5, the reaction can be considered complete upon reaching 2 h reaction time. The work of Tsigie et al. [32] discussed that several distinct stages happen for the entirety of the reaction: subcritical water breaking the rigid cell walls which release intracellular lipids, solvents extracting those lipids, and finally converting the fatty acids into FAMEs.

The effect of reaction time to the power consumed was also similar with temperature. Since the system was not perfectly insulated, the difference of the system and surrounding temperature required significant amount additional energy in order to sustain the system working temperatures for the entirety of the reaction period. Improving the means for insulation can help minimize this, especially if the scale-up of the procedure is to be considered.

3.2.3. Reaction temperature and time

The interaction of the effects of the identified factors is illustrated in Fig. 5. Although the first two factors positively affect the resulting product yield of the process, the severity of the process parameters showed decreased FAME yields when relatively higher temperatures and reaction times were observed simultaneously. Based on Fig. 6, the slight decrease in the obtainable FAME yield began when the reaction temperature and time reaches 230 °C and 2 h, respectively at the same instance. The interactive effects of temperature and time have not been investigated extensively in previous studies, but the general idea as evidenced in literature was that the higher the operating temperature, the faster the reaction will equilibrate in terms of biodiesel conversion, and even faster when using an acid catalyst. For instance, Tsigie et al. [32] considered a temperature of 175 °C and obtained a steady FAME yield starting at the 4th hour with a ratio of 6 ml MeOH per gram of wet biomass. In the work of Go et al. [14], the subcritical reaction can be considered complete after 90 min when they utilized dry samples with an SSR of 10 ml/g at a temperature of 250 °C, down to 60 min when 33% acetic acid by volume was integrated. Tran-Nguyen et al. [31] observed the slight decrease of the yield in an uncatalyzed subcritical in-situ transesterification at 250 °C and more than 2 hrs reaction time and have argued that further extension in the optimum time period may result to reverse transesterification and formation of fatty acids already. Hence under subcritical conditions, the reaction periods must be monitored accordingly when operating at relatively higher temperatures. This scenario, however, was not observed under supercritical conditions [27], and this may be due to the fact that the reaction times were carried out relatively faster than this study. Nevertheless, the working temperatures of supercritical conditions occurring above 280 °C translates to pressures of about 30 MPa [27,44] and therefore necessitates for more expensive pressure vessels required to handle such stress.
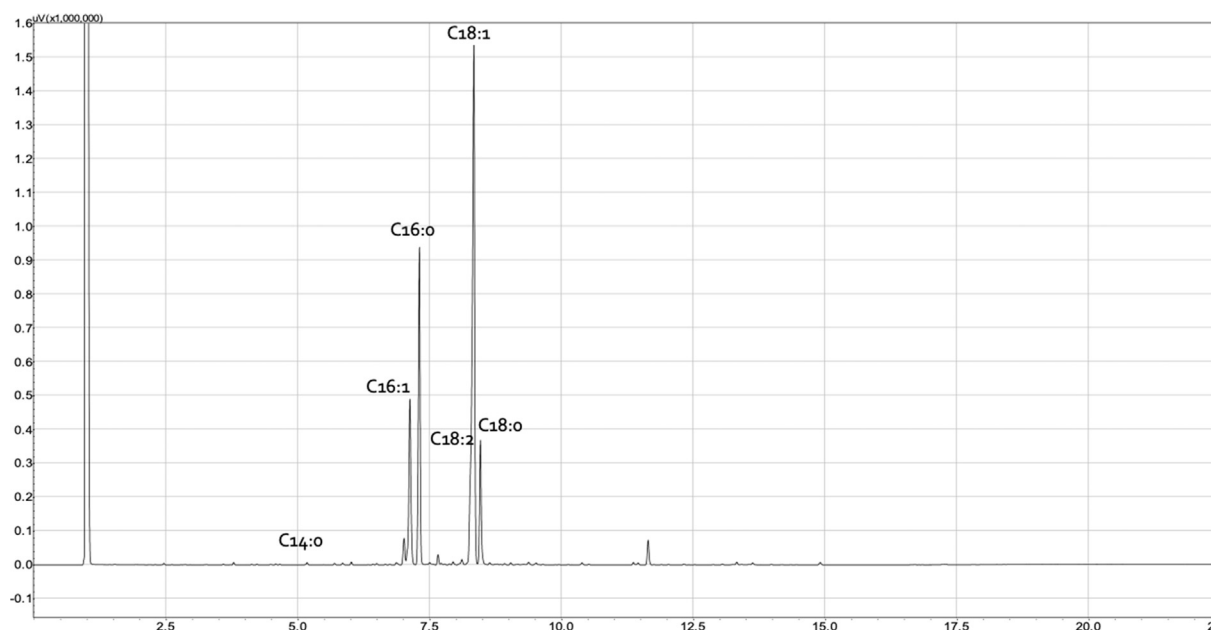
Fig. 4. Chromatogram of fatty acids of *Chlorella vulgaris* biomass under non-catalytic subcritical in-situ transesterification.

Table 3
Definitive screening design of experimental platform with three factors and two responses.

Run	Factors			Responses	
	Temperature (°C)	Time (h)	SSR (ml/g wet basis)	FAME Yield (wt %)	Power consumed (kWh)
1	225	3	8	14.05	0.58
2	225	1	2	5.52	0.34
3	250	2	8	13.74	0.56
4	200	2	2	5.39	0.33
5	250	1	5	10.27	0.49
6	200	3	5	10.51	0.51
7	250	3	2	9.26	0.74
8	200	1	8	7.94	0.33
9	225	2	5	11.49	0.46

3.2.4. Solvent-to-Solid ratio (SSR)

As a solvent and reagent, the amount of methanol directly affects the cost of resources in direct transesterification process [16]. As the reaction is reversible, methanol loading should be in excess especially for uncatalyzed in-situ approaches, but not so much to make the system diluted, lowering the rate of reaction [15]. Typical methanol concentrations of studies utilizing wet algal biomass under subcritical conditions were adapted for this work to make sound comparisons [24,32]. A positive linear trend was observed in the obtainable FAME yield when methanol concentration, with respect to the amount of biomass on a wet basis, was varied accordingly.

Methanol loading had no significant effect on the overall power consumed during the entirety of the process. The small working volume of methanol in the experimental trials (up to 40 ml) may have also been the reason for its insignificance to the power consumed. In addition, methanol has a relatively small heat capacity. Nevertheless, the amount of methanol may become significant upon scaling-up, where working volumes become considerably higher.

3.3. Design of experiment and model evaluation

The results of the DoE trials were fitted with a response surface model, similarly done in the work of Libbrecht et al. [39]. The table of regression coefficients, shown in Table 4, reveals the significance of the main, quadratic, and interaction terms of the three factors on the resulting FAME yield. However, only the main and linear effects of reaction temperature and time was found to have a significant effect on the corresponding power consumption of the process. The regression model also showed goodness of fit in terms of the obtained R^2 values and the trends were consistent with the prior discussion regarding the effects of the identified factors.

Since all of the main terms showed significant effects on the FAME yield, all of the factors were thus considered in getting the optimal operating conditions of the system. Individual maximum desirability functions were assigned to each response which were the FAME yield (maximized) and the process power consumption (minimized). The overall desirability function, which would give the optimum setting of the factor parameters, was then defined based on the geometric mean of the two desirability functions for the individual responses. The optimum operating condition was found to be at approximately 220 °C reaction temperature, 2 h reaction time, and 8 ml methanol per gram of wet biomass, as depicted in Fig. 7. The suggested optimal values by overall maximum desirability and the results of a separate validation experiment show a minimum percent difference in terms of the predicted and actual responses of interest.

Similar to those reported in the related literature, the recommended optimum operating conditions were comparable in terms of the reaction temperature and methanol concentrations, suggesting that the reaction time was the factor that was being altered (decreased) significantly when there was consideration given to the power consumption of the process. This implies that extending the reaction period beyond the optimum value in an effort to slightly improve the product yield may not become favorable due to the increased energy input that will be required by the process itself.

Based on predictive models that are a function of the abundance of fatty acids, several important biodiesel product parameters were estimated and also presented in Table 5. Upon comparison with standards, it was found out that with the exception of cold flow properties, the biodiesel obtainable from the biomass subjected to the proposed method of thermochemical conversion was rather satisfactory.

3.4. Sensitivity analysis

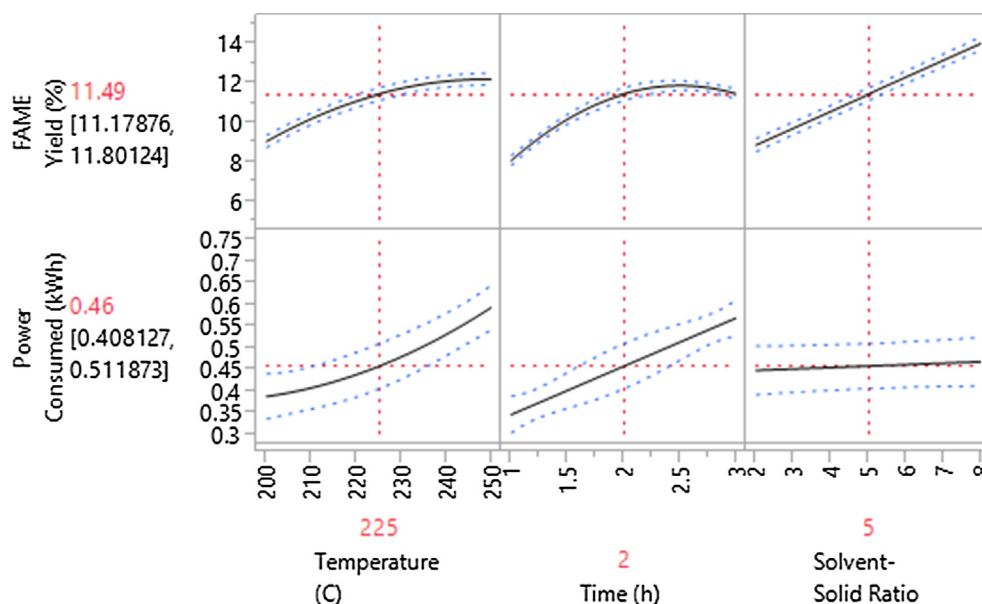Different scenarios which consider the varying importance of

Fig. 5. Prediction profiler of the effects of temperature, reaction time, and solvent-to-solid ratio to the FAME yield and the process power consumption (central treatment values shown).
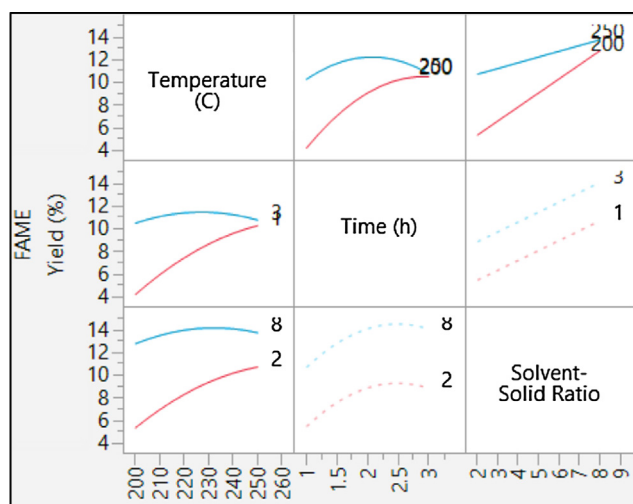

Fig. 6. Factors interaction profiles with their corresponding effects to the FAME yield (broken lines signify that the terms were not found significant).

productivity and costs were evaluated based on the intended distribution of importance in terms of relative weights to FAME yield and power consumption responses. The approach was also implemented

using the JMP software and the optimum conditions for the parameters and the corresponding predicted responses for the generated scenarios were evaluated based on the resulting conditions providing overall maximum desirability for each case. In Table 6, two scenarios (1 and 5) indicate sole importance respectively on either the FAME yield or power consumption, and scenarios 2 and 4 considers both but with slight preference to one response. For each scenario, the optimum settings of the factors and the predicted responses were determined accordingly. The results discussed from the previous section which assumed equal importance for both responses were also listed as scenario 3 to provide comparisons of the results.

The results were somehow straightforward. First, so long as there was weight of importance given to maximizing the FAME yield, the optimum operating temperature would be consistent. When more weight was given to minimizing power consumption, the suggested reaction time becomes essentially lower while the two other factors (temperature and solvent concentration) remained the same. Based on the significance of factors, the reaction time was the most significant factor affecting this response. Considering scenario 5 where all importance was given to power consumption, the model gave the lowest setting for the three input parameters. Scenario 3 was chosen as the best scenario based on the ratio of the FAME yield per kWh as this was deemed a direct and convenient approach in determining which can produce more output (FAME) with less input (power consumption). Although scenario 3 was a compromise having equal importance

Table 4
Regression coefficients of the significant main, interaction, and quadratic effects of factors with their respective *p*-values.

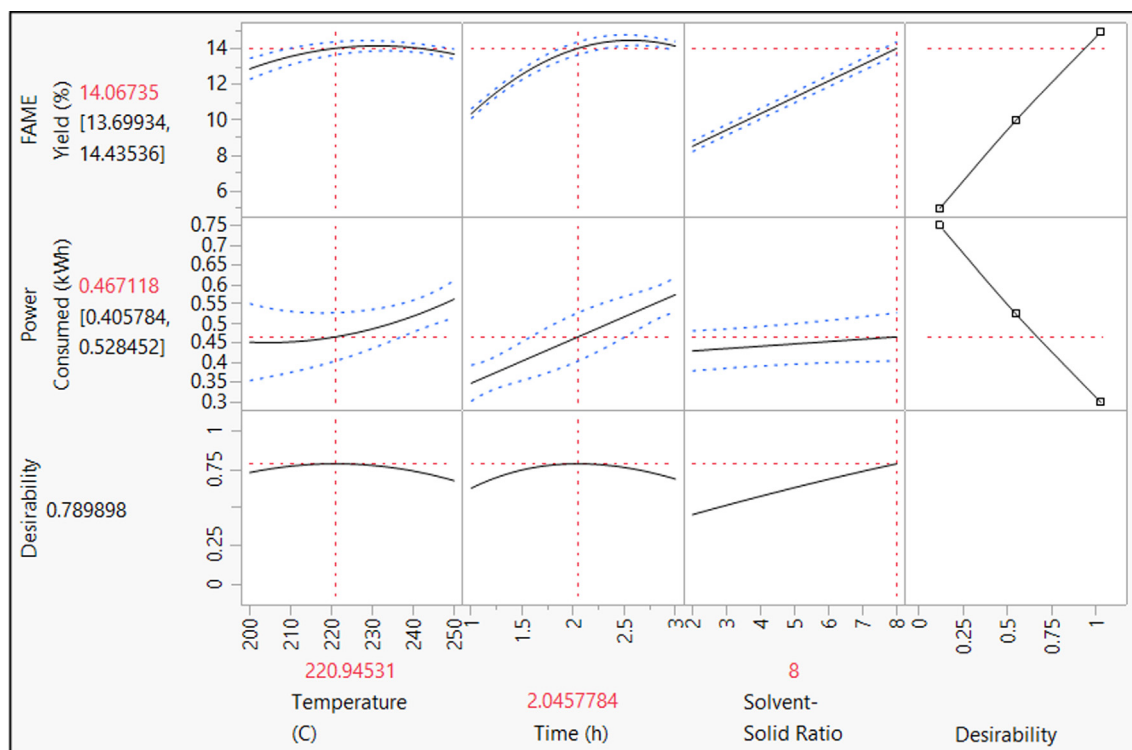| Terms	FAME yield (Y_1)		Power consumed (Y_2)	
	Coeff	*p*-value	Coeff	*p*-value
Intercept	11.49	0.0014	0.482	< 0.0001
Temperature (X_1)	1.571	0.0041	0.1033	0.0011
Time (X_2)	1.682	0.0038	0.1117	0.0007
SSR (X_3)	2.593	0.0025		
Temperature × temperature (X_1^2)	−0.835	0.0132		
Temperature × time (X_1X_2)	−1.44	0.0077		
Time × time (X_2^2)	−1.705	0.0112		
Temperature × SSR (X_1X_3)	−1.09	0.0143		
Model R^2 value	0.9999		0.9262	
Model Prob > F	0.0056		0.0004	

Fig. 7. Predicted optimal values for the three identified factors influencing the FAME yield and process power consumption, with the predicted values of the responses.

between the objectives and consequently having the lowest desirability, it showed the most amount of FAME produced per kWh of power input. In scaling up the process, resource inputs especially related to energy will be of vital importance as this can equally affect the net energy ratios obtained by the process undertaken by the chosen feedstock.

3.5. Comparison of conventional and in-situ process pathways

The sustainability of the in-situ transesterification process has been discussed in many researches. In a review describing different lipid extraction techniques, in-situ transesterification was shown to be comparatively less energy intensive at the expense of lower FAME quality [49]. Karimi [50] did an optimization model of the process with the objective of minimized exergy consumption. Evaluation of the sustainability of this process was also considered by other reports. Tuntiwiwattanapun et al. [51] did a comparative life cycle assessment (LCA) between conventional and in-situ transesterification of spent coffee grounds. Their result yielded a conclusion that the proposed route has less environmental burden than its conventional counterpart.

Uctug et al. [52] also did a comparative LCA between the two processes with microalgae as biomass and similarly concluded that in-situ transesterification had lesser impacts than the other. Lastly, Nezammahallah et al. [53] also did an LCA approach in comparing in-situ transesterification between *Chlorella vulgaris* and *Botryococcous braunii.*

From the literature, LCA has been frequently used in evaluating the sustainability of microalgal biofuels. Aside from the assessment of in-situ transesterification, it has also been used in assessing different bioproducts and bioprocesses such as bio-jet fuels from microalgae [54], thermochemical processing pathways [55], and microalgal biodiesel with co-production [56]. In this work, the researchers employed a simple life-cycle based assessment framework for comparing the impact of conventional transesterification to the proposed in-situ transesterification pathway which subjected the system under subcritical conditions.

The cumulative energy demand (CED) for the conventional pathway was 148.22 MJ per gram of FAME. Meanwhile, the CED of the in-situ pathway was only about one-tenth of the former at 15.68 MJ/g FAME. As illustrated in Fig. 8, the conventional pathway had a mix of energy

Table 5

Summary of key biodiesel properties for *Chlorella vulgaris* biodiesel with different standards.

Fuel property	*C. vulgaris* (this study)	Philippine standard[b]	European standard[c]	American standard[d]
Cetane number	56.18 ± 0.01[a]	42 (min)	51 (min)	47 (min)
Heating value (MJ/kg)	39.42 ± 0.45[b]	Report	35 (min)	Report
Density at 15 °C (g/cc)	0.8733 ± 0.01[b]	n.s.	0.86–0.90	n.s.
Cold filter plugging point	3.34 ± 1.80[c]	n.s.	n.s.	n.s.
Cloud point (°C)	7.84 ± 1.92[c]	5 (max)	n.s.	Report
Kinematic viscosity (mm^2/s) at 40 °C	4.39 ± 0.06[d]	2.4–5	3.5–5	1.9–6

*n.s. – not specified.

[a] Values obtained by adapting several predictive models: Cetane Number [45], Heating Value and Density [46], Cold Filter Plugging and Cloud Point [47], Kinematic Viscosity [48].

[b] Based from B100 specification, obtained by actual experimentation using proposed methods.

[c] Based from EN 14214 specification, obtained by actual experimentation using proposed methods.

[d] Based from ASTM D6751 specification, obtained by actual experimentation using proposed methods.

Table 6
Sensitivity analysis on the effects of varied importance of the FAME yield and process power consumption.

Scenario	Relative importance (%)		Predicted optimum parameter settings			Desirability	Predicted responses		Yield per kWh
	FAME yield (Max)	Power consumed (Min)	Temp (°C)	Time (h)	SSR (ml/g)		FAME Yield (%)	Power consumed (kWh)	
1	100	0	220.47	2.57	8	0.935	14.52	0.53	27.66
2	75	25	220.47	2.20	8	0.817	14.29	0.48	29.52
3	50	50	220.47	2.05	8	0.744	14.07	0.47	30.12
4	25	75	220.47	1.08	8	0.744	10.66	0.36	29.95
5	0	100	200	1	2	0.998	0.57	0.21	2.69
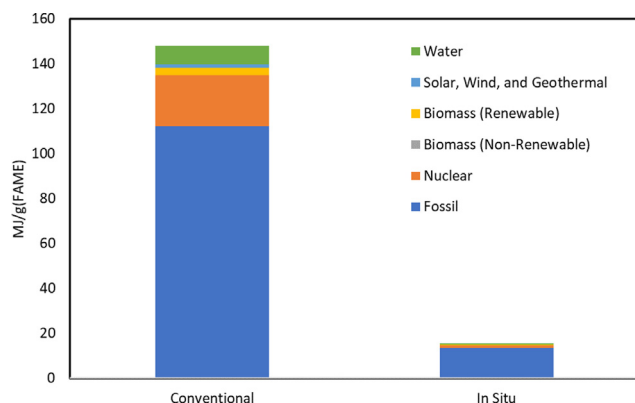
Fig. 8. Cumulative energy demand between conventional and in-situ (sub-critical conditions) pathways.

Table 7
Cumulative energy demand (fossil-based) per input stream of the conventional and in-situ pathways.

| Material stream	Conventional (MJ/g FAME)	In-situ (subcritical) (MJ/g FAME)
Methanol	6.306	7.371
Methoxide	0.329	0.000
Hexane	3.315	0.000
Electricity	102.231	5.994
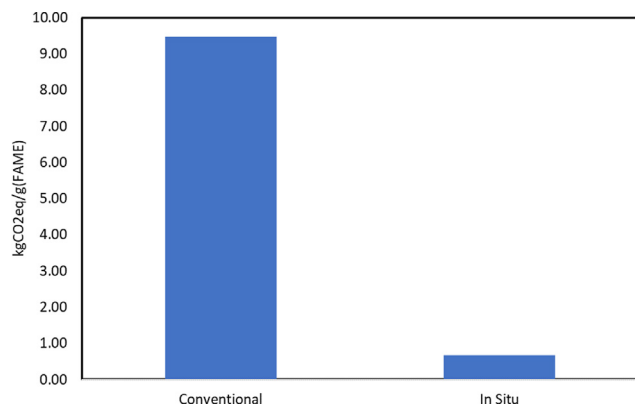
Fig. 9. Global warming potential between conventional and in-situ (subcritical conditions) pathways.

from both non-renewable and renewable sources while the in-situ pathway was derived mainly from fossil fuel. The breakdown of the sources of energy from fossil fuels was enumerated in Table 7. Energy demand from the conventional and in-situ pathway derived 91.13% and 44.85% from electricity consumption, respectively. This indicated that the energy demand for the in-situ pathway was less sensitive to electricity as compared to the conventional route. Improvements in the energy mix can have a greater effect on the conventional pathway.

Table 8
Global warming potential per input stream of the conventional and in-situ pathways.

Material Stream	Conventional (kg CO_2 eq)	In-situ (subcritical) (kg CO_2 eq)
Methanol	0.108	0.545
Methoxide	0.016	0.000
Hexane	0.050	0.000
Electricity	9.300	0.126
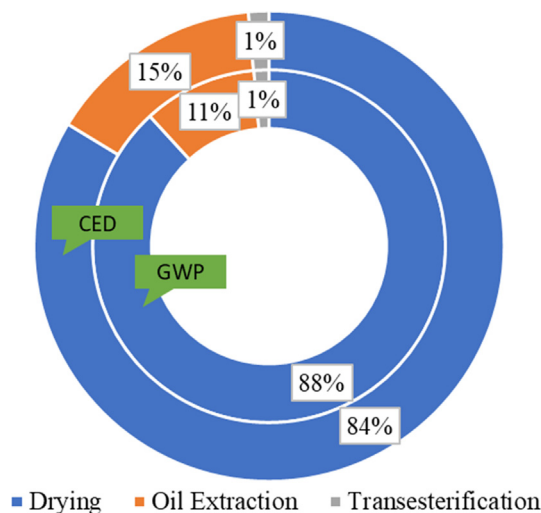
Fig. 10. Process contribution of the conventional pathway for cumulative energy demand (CED) and global warming potential (GWP).

However, it was also determined that 55.15% of the energy demand for in-situ transesterification was associated with methanol consumption. For the compared approaches, the reduction of the overall energy demand would require improvements on both methanol and electricity production.

The results from the global warming potential (GWP) assessment indicated that 1 g of FAME would produce 9.47 kg CO_2 equivalent for conventional pathway and 0.67 kg CO_2 equivalent for the in-situ pathway. These are shown in Fig. 9 and the detailed contribution of each material stream to produce these impacts is shown in Table 8. The results showed 98.16% of the conventional pathway's GWP impact was from electricity consumption while for the in-situ pathway, electricity consumption accounted for only 18.83%. Reduction in electricity consumption had more impact on the conventional pathway than the in-situ pathway. Majority of the in-situ pathway's GWP impact was caused by its methanol consumption, accounting 81.17% of the total impact. Reduction of the in-situ pathway must therefore give emphasis on reducing the methanol consumption as well.

Electricity consumption was the main contributor to the conventional pathway's impact in both CED and GWP. This was reflected on each process' contribution to the impacts. Drying has been identified to have highest impact in the life cycle, as shown in Fig. 10. Therefore,

avoiding this process, such as in the proposed in-situ transesterification route, has provided a significant benefit to the impacts of the compared pathways.

The results of the impact assessment showed that the in-situ transesterification route obtained significantly lesser impact scores compared to the conventional pathway. Overall, the recorded impacts from the two assessments concluded that subcritical in-situ transesterification was far more sustainable and thus more preferable as far as lab-scale processes are concerned. It is also anticipated that upon scaling up, the proposed in-situ pathway would still be relatively less energy demanding and more environmentally friendly than the conventional transesterification counterpart. The reduction in the process steps far outweigh the cost of more resource inputs and the severity of process parameters as far as savings in the overall energy consumption and reduction of environmental footprints are concerned. It is also worth pointing out that the nature of the in-situ route is far more versatile due to its capability to utilize a wide variety of feedstock with high free fatty acid content at a process that does not require the use and post-process removal of certain catalysts.

4. Conclusions and recommendations

This work investigated a non-catalytic in-situ transesterification of *Chlorella vulgaris* with moisture as high as 80 wt%. With the use of a relatively new and more simplified experimental design platform, the effects of key important process parameters were evaluated and were in agreement with related literatures. The optimal conditions of 220 °C, 2 h reaction time, and 8 ml methanol per gram of wet biomass were obtained by not only considering the fatty acid methyl ester (FAME) yield, which was a typical response elsewhere, but also the overall power consumption of the process. These provide a FAME yield of up to 14.07% by weight with respect to the dry biomass (74.6% if with respect to the maximum theoretical FAME obtainable) at a process power consumption rate of 0.47 kWh. The fatty acid profile of the product obtained under the proposed operating conditions also show satisfactory biodiesel properties using various published predictive models.

The study also assessed the global warming potential (GWP) and cumulative energy demand (CED) between conventional transesterification and the proposed in-situ transesterification under subcritical conditions. Results have indicated that the latter had significantly lesser environmental impact and substantial energy savings. This is in parallel with the results from existing literature on the comparative performance between the two biodiesel conversion pathways. In the assessment performed, it was found that electricity consumption was not the sole significant factor of the in-situ pathway's environmental impact. Electricity and methanol consumption have a near equal effect to the CED impact while methanol consumption was the major contributor to its GWP. Therefore, improvements to the proposed in-situ pathway will significantly depend on the manufacturing process or the source of the solvent itself.

The economical aspect of the non-catalytic subcritical in-situ transesterification has a significant influence in the scale-up and commercialization of the technology and must be further investigated in detail. The optimization aspect of this work could also become more comprehensive by considering many other indirect factors. These include reactor loading, operating modes (whether batch or continuous processing), and type of mixing employed. When optimized, they will also translate to significant improvements in the proposed in-situ pathway.

Lastly, since the comparison of the conventional and in-situ transesterification routes was also limited to lab-scale data, many important processes of scaling-up have not been evaluated accordingly. In a biorefinery set-up, this includes the acquisition and transport of raw and intermediate materials, production of potential co-products, as well as product refining, solvents recovery and recycling steps. These scaling effects may also influence the actual energy demand and economics of

both processes which necessitates for pilot-scale investigations. Nevertheless, lab-scale results indicate the potentials of a less energy-intensive and environmentally-friendly process by employing ISTE under subcritical conditions of the solvents.

References

[1] Arnold M, Tainter JA, Strumsky D. Productivity of innovation in biofuel technologies. Energy Policy 2019;124:54–62. https://doi.org/10.1016/j.enpol.2018.09.005.

[2] Suganya T, Varman M, Masjuki HH, Renganathan S. Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: a biorefinery approach. Renew Sustain Energy Rev 2016;55:909–41. https://doi.org/10.1016/j.rser.2015.11.026.

[3] Im HJ, Lee HS, Park MS, Yang JW, Lee JW. Concurrent extraction and reaction for the production of biodiesel from wet microalgae. Bioresour Technol 2014;152:534–7. https://doi.org/10.1016/j.biortech.2013.11.023.

[4] Koller M, Muhr A, Braunegg G. Microalgae as versatile cellular factories for valued products. Algal Res 2014;6:52–63. https://doi.org/10.1016/j.algal.2014.09.002.

[5] Manrique R, Moreno JL, Villagracia AR, Ubando A, Kasai H, Arboleda N, et al. Effects of salinity on the CO2 permeation across lipid bilayer for microalgae biofixation: a molecular dynamics study. J Appl Phycol 2018;30:55–61. https://doi.org/10.1007/s10811-017-1156-9.

[6] Tan XB, Lam MK, Uemura Y, Lim JW, Wong CY, Lee KT. Cultivation of microalgae for biodiesel production: a review on upstream and downstream processing. Chinese J Chem Eng 2018;26:17–30. https://doi.org/10.1016/j.cjche.2017.08.010.

[7] Jiang L, Luo S, Fan X, Yang Z, Guo R. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO2. Appl Energy 2011;88:3336–41. https://doi.org/10.1016/j.apenergy.2011.03.043.

[8] Chen J, Li J, Dong W, Zhang X, Tyagi RD, Drogui P, et al. The potential of microalgae in biodiesel production. Renew Sustain Energy Rev 2018;90:336–46. https://doi.org/10.1016/j.rser.2018.03.073.

[9] Demirbas A. Biodiesel from oilgae, biofixation of carbon dioxide by microalgae: a solution to pollution problems. Appl Energy 2011;88:3541–7. https://doi.org/10.1016/j.apenergy.2010.12.050.

[10] Adeniyi OM, Azimov U, Burluka A. Algae biofuel: current status and future applications. Renew Sustain Energy Rev 2018;90:316–35. https://doi.org/10.1016/j.rser.2018.03.067.

[11] Singh A, Olsen SI. A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. Appl Energy 2011;88:3548–55. https://doi.org/10.1016/j.apenergy.2010.12.012.

[12] Amaro HM, Guedes AC, Malcata FX. Advances and perspectives in using microalgae to produce biodiesel. Appl Energy 2011;88:3402–10. https://doi.org/10.1016/j.apenergy.2010.12.014.

[13] Islam MA, Heimann K, Brown RJ. Microalgae biodiesel: current status and future needs for engine performance and emissions. Renew Sustain Energy Rev 2017;79:1160–70. https://doi.org/10.1016/j.rser.2017.05.041.

[14] Go AW, Sutanto S, Liu YT, Nguyen PLT, Ismadji S, Ju YH. In situ transesterification of Jatropha curcas L. seeds in subcritical solvent system. J Taiwan Inst Chem Eng 2014;45:1516–22. https://doi.org/10.1016/j.jtice.2014.01.010.

[15] Go AW, Sutanto S, Ong LK, Tran-Nguyen PL, Ismadji S, Ju YH. Developments in in-situ (trans)esterification for biodiesel production: a critical review. Renew Sustain Energy Rev 2016;60:284–305. https://doi.org/10.1016/j.rser.2016.01.070.

[16] Park JY, Park MS, Lee YC, Yang JW. Advances in direct transesterification of algal oils from wet biomass. Bioresour Technol 2015;184:267–75. https://doi.org/10.1016/j.biortech.2014.10.089.

[17] Al-lwayzy SH, Yusaf T, Al-Juboori RA. Biofuels from the fresh water microalgae chlorella vulgaris (FWM-CV) for diesel engines. Energies 2014;7:1829–51. https://doi.org/10.3390/en7031829.

[18] Atadashi IM, Aroua MK, Abdul Aziz AR, Sulaiman NMN. The effects of catalysts in biodiesel production: a review. J Ind Eng Chem 2013;19:14–26. https://doi.org/10.1016/j.jiec.2012.07.009.

[19] Kim B, Chang YK, Lee JW. Efficient solvothermal wet in situ transesterification of Nannochloropsis gaditana for biodiesel production. Bioprocess Biosyst Eng 2017;40:723–30. https://doi.org/10.1007/s00449-017-1738-6.

[20] Li Y, Lian S, Tong D, Song R, Yang W, Fan Y, et al. One-step production of biodiesel from Nannochloropsis sp. on solid base Mg-Zr catalyst. Appl Energy 2011;88:3313–7. https://doi.org/10.1016/j.apenergy.2010.12.057.

[21] Kim B, Park J, Son J, Lee JW. Catalyst-free production of alkyl esters from microalgae via combined wet in situ transesterification and hydrothermal liquefaction (iTHL). Bioresour Technol 2017;244:423–32. https://doi.org/10.1016/j.biortech.

2017.07.129.

[22] Martinez-Guerra E, Gude VG, Mondala A, Holmes W, Hernandez R. Microwave and ultrasound enhanced extractive-transesterification of algal lipids. Appl Energy 2014;129:354–63. https://doi.org/10.1016/j.apenergy.2014.04.112.

[23] Martínez N, Callejas N, Morais EG, Vieira Costa JA, Jachmanián I, Vieitez I. Obtaining biodiesel from microalgae oil using ultrasound-assisted in-situ alkaline transesterification. Fuel 2017;202:512–9. https://doi.org/10.1016/j.fuel.2017.04.040.

[24] Sitthithanaboon W, Reddy HK, Muppaneni T, Ponnusamy S, Punsuvon V, Holguim F, et al. Single-step conversion of wet Nannochloropsis gaditana to biodiesel under subcritical methanol conditions. Fuel 2015;147:253–9. https://doi.org/10.1016/j.fuel.2015.01.051.

[25] Patil PD, Gude VG, Mannarswamy A, Cooke P, Nirmalakhandan N, Lammers P, et al. Comparison of direct transesterification of algal biomass under supercritical methanol and microwave irradiation conditions. Fuel 2012;97:822–31. https://doi.org/10.1016/j.fuel.2012.02.037.

[26] Reddy HK, Muppaneni T, Patil PD, Ponnusamy S, Cooke P, Schaub T, et al. Direct conversion of wet algae to crude biodiesel under supercritical ethanol conditions. Fuel 2014;115:720–6. https://doi.org/10.1016/j.fuel.2013.07.090.

[27] Jazzar S, Olivares-Carrillo P, Pérez de los Ríos A, Marzouki MN, Acién-Fernández FG, Fernández-Sevilla JM, Molina-Grima E, Smaali I, Quesada-Medina J. Direct supercritical methanolysis of wet and dry unwashed marine microalgae (Nannochloropsis gaditana) to biodiesel. Appl Energy 2015;148:210–9. https://doi.org/10.1016/j.apenergy.2015.03.069.

[28] Ju YH, Huynh LH, Tsigie YA, Ho QP. Synthesis of biodiesel in subcritical water and methanol. Fuel 2013;105:266–71. https://doi.org/10.1016/j.fuel.2012.05.061.

[29] Go AW, Tran Nguyen PL, Huynh LH, Liu YT, Sutanto S, Ju YH. Catalyst free esterification of fatty acids with methanol under subcritical condition. Energy 2014;70:393–400. https://doi.org/10.1016/j.energy.2014.04.013.

[30] Gunawan F, Kurniawan A, Gunawan I, Ju YH, Ayucitra A, Soetaredjo FE, et al. Synthesis of biodiesel from vegetable oils wastewater sludge by in-situ subcritical methanol transesterification: process evaluation and optimization. Biomass Bioenergy 2014;69:28–38. https://doi.org/10.1016/j.biombioe.2014.07.005.

[31] Tran-Nguyen PL, Go AW, Ismadji S, Ju YH. Transesterification of activated sludge in subcritical solvent mixture. Bioresour Technol 2015;197:30–6. https://doi.org/10.1016/j.biortech.2015.08.033.

[32] Tsigie YA, Huynh LH, Ismadji S, Engida AM, Ju YH. In situ biodiesel production from wet Chlorella vulgaris under subcritical condition. Chem Eng J 2012;213:104–8. https://doi.org/10.1016/j.cej.2012.09.112.

[33] Zhao J, Li W, Qu H, Tian G, Wei Y. Application of definitive screening design to quantify the effects of process parameters on key granule characteristics and optimize operating parameters in pulsed-spray fluid-bed granulation. Particuology 2018. https://doi.org/10.1016/j.partic.2018.03.007.

[34] Malekzadeh M, Abedini Najafabadi H, Hakim M, Feilizadeh M, Vossoughi M, Rashtchian D. Experimental study and thermodynamic modeling for determining the effect of non-polar solvent (hexane)/polar solvent (methanol) ratio and moisture content on the lipid extraction efficiency from Chlorella vulgaris. Bioresour Technol 2016;201:304–11. https://doi.org/10.1016/j.biortech.2015.11.066.

[35] Tran Nguyen PL, Go AW, Huynh LH, Ju YH. A study on the mechanism of subcritical water treatment to maximize extractable cellular lipids. Biomass Bioenergy 2013;59:532–9. https://doi.org/10.1016/j.biombioe.2013.08.031.

[36] Salam KA, Velasquez-Orta SB, Harvey AP. A sustainable integrated in situ transesterification of microalgae for biodiesel production and associated co-products – a review. Renew Sustain Energy Rev 2016;65:1179–98. https://doi.org/10.1016/j.rser.2016.07.068.

[37] Jones B, Nachtsheim CJ. A class of three-level designs for definitive screening in the presence of second-order effects. J Qual Technol 2011;43:1–15. https://doi.org/10.1080/00224065.2011.11917841.

[38] Felix C, Ubando A, Madrazo C, Culaba A, Go AW, Sutanto S, Ju YH, Tran-Nguyen PL, Chang JS. Uncatalyzed direct biodiesel production from wet microalgae under subcritical conditions. In: HNICEM 2017 – 9th Int. Conf. Humanoid, Nanotechnology, Inf. Technol. Commun. Control. Environ. Manag., vol. 2018–January, 2018. https://doi.org/10.1109/HNICEM.2017.8269551.

[39] Libbrecht W, Deruyck F, Poelman H, Verberckmoes A, Thybaut J, De Clercq J, et al. Optimization of soft templated mesoporous carbon synthesis using Definitive Screening Design. Chem Eng J 2015;259:126–34. https://doi.org/10.1016/j.cej.2014.07.113.

[40] Ahmad F, Khan AU, Yasar A. Transesterification of oil extracted from different species of algae for biodiesel production. African J Environ Sci Technol 2013;7:358–64. https://doi.org/10.5897/AJEST12.167.

[41] Chaudry S, Bahri PA, Moheimani NR. Life cycle analysis of milking of microalgae for renewable hydrocarbon production. Comput Chem Eng 2019;121:510–22. https://doi.org/10.1016/j.compchemeng.2018.11.019.

[42] Go AW, Sutanto S, Tran-Nguyen PL, Ismadji S, Gunawan S, Ju YH. Biodiesel production under subcritical solvent condition using treated whole Jatropha curcas seed kernels and possible use of hydrolysates to grow Yarrowia lipolytica. Fuel 2014;120:46–52. https://doi.org/10.1016/j.fuel.2013.11.066.

[43] Huynh LH, Tran Nguyen PL, Ho QP, Ju YH. Catalyst-free fatty acid methyl ester production from wet activated sludge under subcritical water and methanol condition. Bioresour Technol 2012;123:112–6. https://doi.org/10.1016/j.biortech.2012.08.001.

[44] Jazzar S, Quesada-Medina J, Olivares-Carrillo P, Marzouki MN, Acién-Fernández FG, Fernández-Sevilla JM, et al. A whole biodiesel conversion process combining isolation, cultivation and in situ supercritical methanol transesterification of native microalgae. Bioresour Technol 2015;190:281–8. https://doi.org/10.1016/j.biortech.2015.04.097.

[45] Piloto-Rodríguez R, Sánchez-Borroto Y, Lapuerta M, Goyos-Pérez L, Verhelst S. Prediction of the cetane number of biodiesel using artificial neural networks and multiple linear regression. Energy Convers Manag 2013;65:255–61. https://doi.org/10.1016/j.enconman.2012.07.023.

[46] Ramírez-Verduzco LF, Rodríguez-Rodríguez JE, Jaramillo-Jacob ADR. Predicting cetane number, kinematic viscosity, density and higher heating value of biodiesel from its fatty acid methyl ester composition. Fuel 2012;91:102–11. https://doi.org/10.1016/j.fuel.2011.06.070.

[47] Pinzi S, Leiva D, Arzamendi G, Gandia LM, Dorado MP. Multiple response optimization of vegetable oils fatty acid composition to improve biodiesel physical properties. Bioresour Technol 2011;102:7280–8. https://doi.org/10.1016/j.biortech.2011.05.005.

[48] Knothe G. "Designer" biodiesel: optimizing fatty ester composition to improve fuel properties. Energy Fuels 2008;22:1358–64. https://doi.org/10.1021/ef700639e.

[49] Jeevan Kumar SP, Vijay Kumar G, Dash A, Scholz P, Banerjee R. Sustainable green solvents and techniques for lipid extraction from microalgae: a review. Algal Res 2017;21:138–47. https://doi.org/10.1016/j.algal.2016.11.014.

[50] Karimi M. Exergy-based optimization of direct conversion of microalgae biomass to biodiesel. J Clean Prod 2017;141:50–5. https://doi.org/10.1016/j.jclepro.2016.09.032.

[51] Tuntiwiwattanapun N, Usapein P, Tongcumpou C. The energy usage and environmental impact assessment of spent coffee grounds biodiesel production by an in-situ transesterification process. Energy Sustain Dev 2017;40:50–8. https://doi.org/10.1016/j.esd.2017.07.002.

[52] Uctug G, Modi DN, Mavituna F. Life cycle assessment of biodiesel production from microalgae: a mass and energy balance approach in order to compare conventional with in situ transesterification. Int J Chem Eng Appl 2017;8:355–6. https://doi.org/10.18178/ijcea.2017.8.6.683.

[53] Nezammahalleh H, Adams TA, Ghanati F, Nosrati M, Shojaosadati SA. Techno-economic and environmental assessment of conceptually designed in situ lipid extraction process from microalgae. Algal Res 2018;35:547–60. https://doi.org/10.1016/j.algal.2018.09.025.

[54] Fortier MOP, Roberts GW, Stagg-Williams SM, Sturm BSM. Life cycle assessment of bio-jet fuel from hydrothermal liquefaction of microalgae. Appl Energy 2014;122:73–82. https://doi.org/10.1016/j.apenergy.2014.01.077.

[55] Bennion EP, Ginosar DM, Moses J, Agblevor F, Quinn JC. Lifecycle assessment of microalgae to biofuel: comparison of thermochemical processing pathways. Appl Energy 2015;154:1062–71. https://doi.org/10.1016/j.apenergy.2014.12.009.

[56] Gnansounou E, Kenthorai Raman J. Life cycle assessment of algae biodiesel and its co-products. Appl Energy 2016;161:300–8. https://doi.org/10.1016/j.apenergy.2015.10.043.